applied microbiology

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Growth and Metabolism of *Clostridioides difficile* in Hungate-Style Media

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Abstract: *Clostridioides difficile* is a clinically and agriculturally important organism with diverse metabolic capabilities. Commercially available media types to cultivate *C. difficile* typically include multiple growth substrates and often selective agents. Under these conditions, it is difficult to determine what the bacteria utilized and which products are derived from which substrates. These experiments compared a commercial broth (Reinforced Clostridium Medium/RCM) to simpler, defined, carbonate-based media types influenced by Robert Hungate. Peptides (tryptone peptone), amino acids (casamino acids), and/or glucose were added to evaluate the growth of *C. difficile* strains 9689, BAA-1870, and 43597, and the metabolism of the type strain 9689. *C. difficile* grew to the greatest optical density in the rich RCM broth but produced less ammonia than the tryptone-containing media types. *C. difficile* utilized all glucose in RCM and T+G media in addition to performing amino acid fermentations, though the volatile fatty acids produced were not necessarily consistent across media type. When cultured in CAA-containing media, 9689 performed very little metabolism and did not grow regardless of supplementation with glucose. These data demonstrated that *C. difficile* could metabolize substrates and grow in defined, anaerobic, and carbonate-buffered media. Hungate-style media appear to be an acceptable choice for reliable culturing of *C. difficile*.

Keywords: *Clostridioides difficile*; metabolism; Hungate-style media; commercial broth

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Citation: Lakes, J.E.; Ferrell, J.L.; Flythe, M.D. Growth and Metabolism of *Clostridioides difficile* in Hungate-Style Media. *Appl. Microbiol.* **2024**, *4*, 85–95. https://doi.org/10.3390/applmicrobiol4010006

Academic Editor: Ian Connerton

Received: 8 November 2023
Revised: 22 December 2023
Accepted: 25 December 2023
Published: 30 December 2023

1. Introduction

Clostridioides difficile is an anaerobic, spore-forming, Gram (+), and zoonotic bacterium that has a well-established history as a human gastrointestinal (GI) pathogen, but it also greatly impacts numerous animal species [1,2]. While its primary habitat is the GI tract of humans and animals, it was also isolated from soil systems, various sources of water (sometimes as the result of contamination), as well as in crops/crop fields [3–6]. *C. difficile* was first isolated from the fecal material of newborn infants in 1935 by Hall and O'Toole and was originally named for its shape (rod—Bacillus) and difficult culture requirements (difficilis) [7]. This microorganism has since been renamed once and reclassified twice: first to *Clostridium difficile* [8] and then to its current designation, *Clostridioides difficile* [9].

Traditionally, the study of *C. difficile* has been conducted with interest in its pathogenic capabilities, leading to the importance of quick isolation and detection of the bacterium in human and animal populations as well as potential sources of environmental and food contamination [6,10–12]. As such, *C. difficile* culture media tend to be selective and commercially manufactured. In clinical settings, sampling for the presence of *C. difficile* in symptomatic patients or hospital environments (fecal samples or swab materials) often relies on the use of pre-made selective agar plates or broths such as cycloserine cefoxitin fructose agar (CCFA), sodium taurocholate-enriched cycloserine-cefoxitin-amphotericin B (TCCA), and fructose agar (TCCFA), Columbia blood agar, fastidious agar (FA) for spore collection, or *Clostridium difficile* moxalactam norfloxacin agar (CDMN) [12,13].

Media for *C. difficile* isolation or enrichment from food testing were largely adapted from the culture techniques used in clinical settings and isolations [10,14,15]. CDMN broth supplemented with 0.1% (*w*/*v*) sodium taurocholate (TCDMN) and brain–heart infusion (BHI) broths supplemented with 0.1% (*w*/*v*) sodium taurocholate (TBHI), D-cycloserine, and cefoxitin (TBHICC) are two of the more commonly used enrichment broths in the testing of meat products for *C. difficile* [16]. Pre-made agars are regularly utilized for the isolation of *C. difficile* in potentially contaminated meat products: CDMN agar, *Clostridioides difficile* selective supplement with D-cycloserine, cefoxitin, neutral red (CCFN), and 0.1% (*w*/*v*) sodium taurocholate (TCCFN) [16].

Pre-made broths and agars are sufficient when testing for the presence or absence of *Clostridioides difficile* in clinical or industrial samples. However, rich media with multiple growth substrates and selective agents are not ideal for metabolic studies. It can be advantageous to design the culture medium (broth or agar) with defined substrates to test *C. difficile*'s versatile metabolic capabilities. *C. difficile* readily performs amino acid fermentations via Stickland reactions, can utilize sugars and other carbohydrates through an incomplete Krebs cycle, and can utilize the Wood–Ljungdahl pathway where CO_2 can be reduced to acetate, regenerating an NAD^+ in the process [17].

Rumen microbiologists often rely on custom media onto which substrates reflective of a diet can be added. Diet affects the gastrointestinal flora. From previous cattle studies, it was demonstrated that feeding differing substrates (dietary components) alter the microbial composition of the rumen. For example, grazing or forage-fed cattle whose primary source of nutrition comes from grasses and other forages have a ruminal composition to reflect that substrate source, i.e., high levels of fibrolytic and cellulolytic bacteria [18–20]. Whereas typical American finishing cattle on a high starch diet (e.g., corn) have a different microbial profile than these same cattle when they were feeding on grasses, i.e., large increases in *Prevotella* and *Streptococcus* species coupled with marked decreases in most fibrolytic bacteria [19,20]. The dietary changes culminate in the production of different microbial metabolic products concomitantly with compositional changes, namely, in lactate and propionate production [18]. Customizable, defined, liquid media allow determination of product formation from specific substrates.

Our laboratory cultures bacteria in custom media based on the work of Robert Hungate and subsequent rumen microbiologists. The media are thermally degassed, cooled under carbon dioxide, and carbonate-buffered. A series of simple growth and metabolic experiments were undertaken to determine (1) Will Hungate-style media support the growth of *C. difficile*? (2) What metabolic products can be detected in this broth type when one or more common growth substrates are included? (3) How do growth and metabolism in the defined medium compare to a common commercial medium? The data reported and discussed here are intended to serve as a foundation for bacterial physiologists. The work demonstrates the value of controlling substrate availability within the media for the study of bacterial metabolism using a few well-characterized strains of a pathogen of interest, *Clostridioides difficile*.

2. Materials and Methods

2.1. Strains

Clostridioides difficile type strain ATCC 9689 (strain 90556-M6S; ribotype 001; toxinotype 0—*tcdA* and *tcdB* positive), BAA-1870 (strain 4118; ribotype 027; toxinotype IIIb—*ctdB*, *tcdA*, and *tcdB* positive), and ATCC 43597 (strain VPI 11186; ribotype 014; toxinotype 0—*tcdA* and *tcdB* positive) were obtained from the American Type Culture Collection—ATCC (Manassas, VA, USA) as freeze-dried culture stocks. All cultures were routinely examined for contamination by Gram stain and wet mount light microscopy using a Motic light microscope (Hong Kong, China) (Gram-positive, distended rods, endospores, etc.). They were also checked for production of expected products by HPLC per the section below.

2.2. Growth Media Composition

Reinforced clostridium medium (RCM) was prepared (per 1 L) per manufacturer instructions (BD Difco™, Franklin Lakes, NJ, USA). Briefly, 38 g RCM powder was dissolved into 1 L boiling deionized H_2O and cooled under N_2. The approximate medium composition as defined by the manufacturer per liter is 10 g peptone, 10 g beef extract, 3 g yeast extract, 5 g dextrose, 5 g NaCl, 1 g soluble starch, 500 mg cysteine HCl, and 3 g sodium acetate at pH 6.8. Cooled medium was anaerobically dispensed into borosilicate serum bottles (52 × 95 mm) and autoclaved (121 °C, 20 min) for sterility. Hungate-style basal medium (BM) was based on [21] and contained (per 1 L): 600 mg cysteine HCl, 240 mg KH_2PO_4, 1 mg $FeSO_4$ •$7H_2O$, 0.05 mg $ZnSO_4$ •$7H_2O$, 240 mg K_2HPO_4, 2 mg CaD pantothenate, 480 mg NaCl, 516 mg Na_2SO_4, 0.05 mg folic acid, 100 mg $MgSO_4$ •$7H_2O$, and 64 mg $CaCl_2$ •$2H_2O$, 2 mg riboflavin, 2 mg thiamine HCl, 0.1 mg $CoCl_2$ •$6H_2O$, 0.1 mg p-aminobenzoic acid, 1 mg lipoic acid, 0.05 mg biotin, 0.05 mg cobalamin, 1 mg pyridoxal HCl, 1 mg pyridoxine, 1 mg pyridoxamine 2HCl, 2.5 mg Na_4EDTA, 2 mg nicotinamide, 1 mg $MnCl_2$ •$4H_2O$, 0.1 mg H_3BO_3, 5 µg $CuCl_2$ •$2H_2O$, 0.01 mg $NiCl_2$ •$6H_2O$, and 0.015 mg $NaMoO_4$ •$2H_2O$. The broth was adjusted to pH 6.5 via addition of NaOH or HCl, then autoclaved to remove O_2 from solution, and cooled under CO_2. After cooling, 4.0 g Na_2CO_3 was added to the medium as a buffer and dispensed into borosilicate serum bottles (52 × 95 mm) and autoclaved for sterility. The prepared BM was supplemented with either trypticase tryptone (15 mg mL^{-1} final concentration; T) (BD Difco™, Franklin Lakes, NJ, USA), trypticase tryptone (15 mg mL^{-1} final concentration) and glucose (2 mg mL^{-1} final concentration; T+G), casamino acids (15 mg mL^{-1} final concentration; CAA) (BD Difco™, Franklin Lakes, NJ, USA), or casamino acids (15 mg mL^{-1} final concentration) and glucose (2 mg mL^{-1} final concentration; CAA+G).

2.3. Growth Experiments

Growth experiments were conducted in 50 mL of each of the growth medium described above (RCM, T, T+G, CAA, and CAA+G) in serum bottles. Each bottle was inoculated (10% v/v) from stationary phase (20 h) cultures, maintained in RCM broth, and incubated at 39 °C [22]. Growth was assessed via optical density (600 nm) measurements using a spectrophotometer (Biochrom WPA Biowave II Life Sciences, Cambridge, Cambridgeshire, United Kingdom). Briefly, 2 mL of sample was collected, and optical density measured every hour for strain 9689, starting immediately after inoculation (0 h), for the next 8 h until the exponential phase was reached, and then a final timepoint was taken 24 h post-inoculation. Optical density in the respective media types for *C. difficile* strains BAA-1870 and 43597 were measured at the 0 and 24 h timepoints only.

2.4. HPLC Metabolite Analysis

Samples were collected concurrently with growth experiment timepoints (0–8, 24 h) and filtered using a 0.22 µm syringe filter then frozen at −20 °C until analysis. The fermentation end products succinate, formate, acetate, butyrate, isobutyrate, lactate, valerate, caproate (hexanoic acid), isocaproate (4-methylvaleric acid), IVMB (isovalerate/methylbutyrate), and p-cresol; as well, glucose utilization were quantified using liquid chromatography (Summit HPLC; Dionex, Sunnyvale, CA, USA) equipped with an anion exchange column (Aminex, HP-87H; Bio-Rad, Hercules, CA, USA) protected by a Micro-Guard Cation H guard column. The eluted compounds were isostatically separated in an aqueous sulfuric acid mobile phase (5 mmol L^{-1}). The column operated at 50 °C with a flow rate of 0.4 mL min^{-1} and injection volume of 50 µL. The detection of compounds was performed by refractive index (ERC Refractomax 520 RI, Prague, Czech Republic) and UV-vis at 210 nm (Thermo, Dionex 3000 Multiwavelength Detector, Waltham, MA, USA) concurrently. Chromeleon™ Chromatography Data System Software v7.2 was used for collection and quantification of the data.

2.5. Ammonia Assay

Samples for ammonia concentration analysis were taken as described above for metabolite analysis. Briefly, 1 mL of sample was extracted from inoculated serum bottles of all media types described, hourly at 0–8 h and 24 h timepoints. Samples were clarified via filtering through a 0.22 μm syringe filter and were stored at −20 °C until the assay could be performed. Ammonia concentration for each sample and control were determined using the phenolic acid/hypochlorite method with absorbance analysis at 630 nm previously described by Chaney and Marbach [23] with 6× concentrated reagents.

2.6. Sporulation

All experiments were conducted in triplicate for each media type. *C. difficile* sporulation experiments were conducted in growth media described above (RCM, T, T+G, CAA, and CAA+G) for all strains in Hungate tubes. All tubes were inoculated (10% v/v) with stationary phase (~20 h) cultures maintained in RCM broth, regularly transferred and incubated at 39 °C. The experimental tubes (RCM, T, T+G, CAA, and CAA+G), post-inoculation, were incubated for the next 48 h at 39 °C as sporulation is generally a stationary phase occurrence in laboratory-maintained cultures. Fresh RCM tubes were inoculated from each experimental tube (10% v/v) then pasteurized at 80 °C for 20 min to destroy the living cells. After cooling to room temperature (~23 °C), the pasteurized tubes were incubated for the next 10 days at 39 °C to determine the impact of substrate type on sporulation.

2.7. Statistics

All experiments were conducted in triplicate for each media type and statistical analyses were conducted utilizing OriginPro statistical software (OriginLab Corporation, Northampton, MA, USA). Metabolite data were analyzed for significant differences in glucose utilized and subsequent metabolites produced between 0 and 24 h measurements using a paired *t*-test. Significant differences in metabolites, including ammonia, produced after 24 h between the five media types (RCM, T, T+G, and CAA+G) were performed utilizing one-way ANOVA followed by Tukey's post hoc test. Data are represented as averages ± SD. Significance was defined as $p < 0.05$.

3. Results

3.1. Growth Media Results

Clostridioides difficile (9689) grew reliably to an optical density (OD_{600}) of 3.0 in RCM media (Figure 1) at a growth rate (μ) of approximately 0.7 h^{-1}. In similar fashion, strains BAA-1870 and 43597 also grew reliably in RCM media (Δ OD of 3.0 and 3.4, respectively). In BM with trypticase added (15 mg mL^{-1}), 9689 grew to an OD_{600} between 0.5 and 1.0 at μ = 0.5 h^{-1}, which was also the case for the other *C. difficile* strains tested. When BM was supplemented with trypticase tryptone (15 mg mL^{-1}) and glucose (2 mg mL^{-1}), 9689 grew at a comparable rate (μ = 0.5 h^{-1}) but to a greater optical density than when grown in media supplemented with trypticase alone (1.5 versus 0.5–1.0). Comparatively, strains BAA-1870 and 43597 grew to even greater optical density in T+G media than 9689 (3.0 and 2.1, respectively). When 9689 was inoculated into media containing casamino acids (CAA) or in media with CAA and glucose (15 mg mL^{-1} and 2 mg mL^{-1}, respectively), it did not grow (μ = 0.0) (Figure 1). Similar to 9689, when *C. difficile* strains BAA-1870 and 43597 were inoculated into media containing CAA or CAA+G, they did not grow (Δ OD of 0.0).

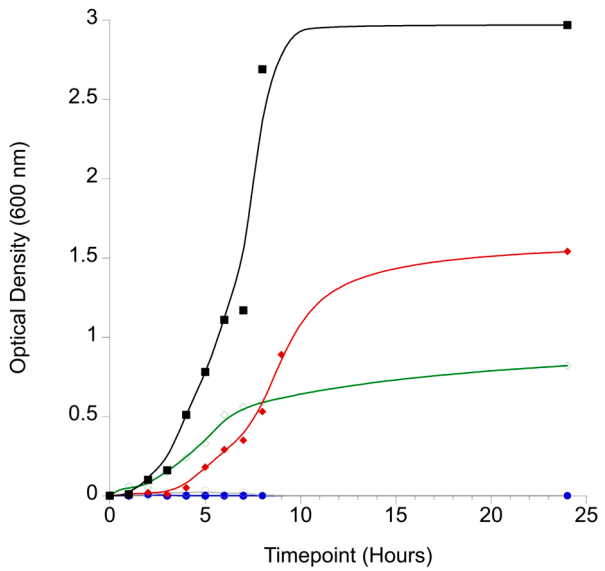


Figure 1. Representative growth of 9689 over 24 h per media type. Growth curves were conducted in triplicate and first-order growth rate kinetics (expressed as h^{-1}) were calculated for each replicate to verify reproducibility. CAA+G (blue filled circles): 0.0 h^{-1}; CAA (gray open circles): 0.0 h^{-1}; T (green open diamonds): 0.5 h^{-1}; T+G (red filled diamonds): 0.5 h^{-1}; and RCM (black filled squares): 0.7 h^{-1}.

3.2. Ammonia Production per Media Type

Ammonia production by *Clostridioides difficile* was measured over a 24 h period of growth as follows: every hour after inoculation until 8 h (0–8 h) and then a 24 h endpoint sample. Overall, 9689 produced the most ammonia when grown in media amended with trypticase (15 mg mL^{-1}), including T and T+G media types (24.7 mM and 18.3 mM ammonia, respectively) (Figure 2). Overall, 9689 produced comparatively less ammonia when grown in RCM and CAA media types (Figure 2). In fact, 9689 produced less than 5 mM ammonia after 24 h of growth in CAA and CAA+G media (Figure 2). Whereas in RCM media, 9689 produced 6.7 mM of ammonia after 24 h of growth.

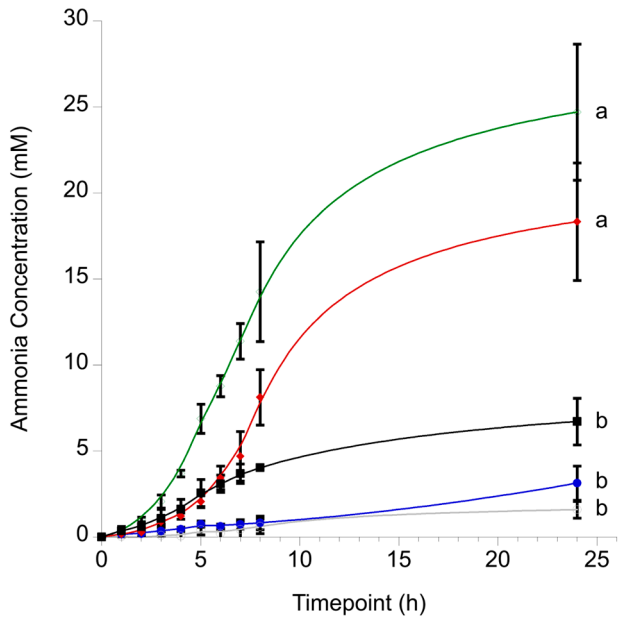


Figure 2. Ammonia production by 9689 over 24 h per media type. Ammonia values are represented as an average of three replicates and error bars are represented as ± standard deviation (SD) values.

Significant differences were determined between the media types at 24 h. CAA+G (blue filled circles); CAA (gray open circles); T (green open diamonds); T+G (red filled diamonds); RCM (black filled squares). Significance is denoted by alphabetical label. Labels with the same alphabetical designation are not statistically different. Significance is defined as $p < 0.05$.

3.3. Fermentation Acid Production per Media Type and the Utilization of Glucose

From inoculation (0 h) to the 24 h endpoint sample, 9689 produced the following fermentation acids in RCM, T, T+G, or CAA+G media types: formate, acetate, butyrate, isovalerate methylbutyrate (IVMB), valerate, and isocaproate (4-methylvaleric acid) (Table 1). In RCM media, *Clostridioides difficile* utilized the glucose, and some of the exogenous acetate, available in the medium and consistently produced formate, butyrate, valerate, and isocaproate at quantifiable concentrations (Figure 3a, Table 1). Additionally, lactate, acetate, succinate, and IVMB were included as standards in the analysis; however, they were not produced at quantifiable levels in RCM media. *C. difficile* produced more formate, butyrate, valerate, and isocaproate after 24 h of growth and metabolism ($p < 0.05$) (Figure 3a, Table 1). In T+G media, 9689 utilized all glucose available in the medium and subsequently produced quantifiable concentrations of formate, acetate, butyrate, IVMB, valerate, and isocaproate over the 24 h sample period (Figure 3b, Table 1). *C. difficile* produced significantly more acetate, butyrate, IVMB, valerate, and isocaproate after 24 h of metabolic activity ($p < 0.05$) (Figure 3b). When 9689 was grown in T media, it produced quantifiable amounts of acetate, IVMB, valerate, and isocaproate (Table 1). Alternatively, in CAA + G media, 9689 produced few metabolites and utilized little glucose (~0.5 mM) over 24 h (Table 1). However, there were increases, though small, in acetate and isocaproate produced from inoculation to 24 h in CAA+G media ($p < 0.05$). *C. difficile* did not appear to perform many metabolic processes once inoculated in CAA media, and therefore did not produce detectable quantities of fermentation acids.

Table 1. Effect of substrate type on fermentation acid production by *Clostridioides difficile* (9689) after 24 h of growth represented as the average of three trials.

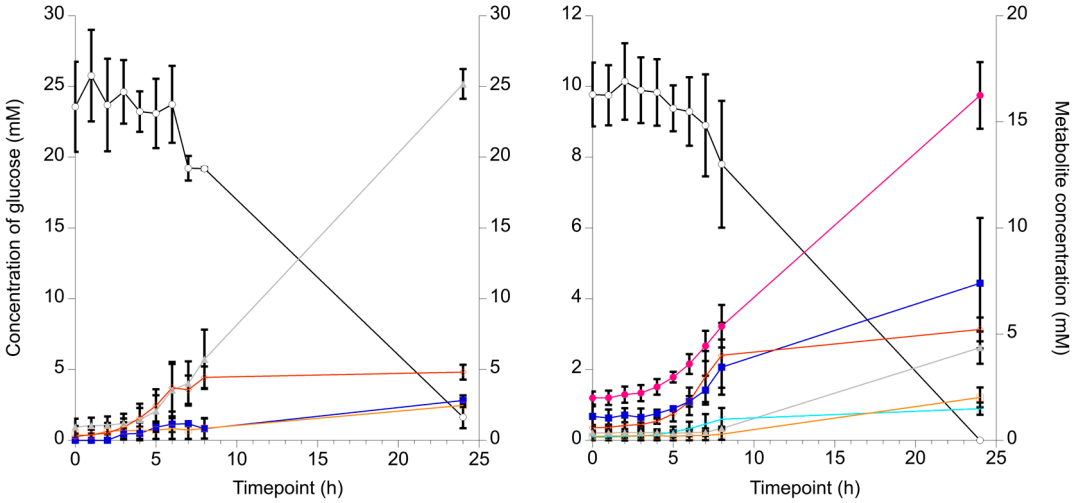
Metabolites	Fermentation Acid Products (mM)			
	RCM	T	T+G	CAA+G
Lactate	NQ	NQ	NQ	NQ
Formate	2.80 ^{a}	NQ	7.40 ^{c}	NQ
Acetate	NQ	7.58 ^{a}	16.24 ^{b}	3.27 ^{c}
Butyrate	25.17 ^{a}	NQ	4.36 ^{b}	NQ
Succinate	NQ	NQ	NQ	NQ
IVMB	NQ	1.99 ^{a}	1.49 ^{a}	NQ
Valerate	2.46 ^{a}	1.45 ^{b}	2.02 ^{a,b}	NQ
Isocaproate	4.81 ^{a}	4.79 ^{a}	5.22 ^{a}	1.32 ^{b}

NQ = not quantifiable. Values with the same superscript letter are statistically the same across a row, $p < 0.05$.

After 24 h of growth and metabolism, 9689 produced more formate and acetate when grown in T+G media than in any of the other media types tested (Table 1). In RCM media, *C. difficile* produced far more butyrate than when grown in any other media type (Table 1). *C. difficile* produced more isocaproate in RCM, T, and T+G media types than it did when grown in CAA+G media (Table 1). Generally, when grown in CAA+G media, *C. difficile* produced less quantifiable fermentation acids than in any other media type with the exception of acetate, which was not quantifiable in RCM media (Table 1).

Next, the efficiency by which *C. difficile* utilized the substrates was determined using a function of ΔOD_{600} per Δtotal catabolic products (mM) it produced in each media type (Table 2). Per every 1 mM total catabolic metabolites produced, 9689 grew approximately 0.066 OD in RCM media. The media types most similar to RCM in efficiency were BM T

and T+G at 0.020 and 0.026 OD per 1 mM products, respectively (Table 2). *C. difficile* was the most inefficient at utilizing the substrates in CAA and CAA+G media types.



(**a**) (**b**)

Figure 3. Glucose utilization and fermentation acid production by 9689 in RCM (**a**) and T+G (**b**) media. Glucose (black open circles), acetate (pink filled circles), formate (blue filled squares), butyrate (gray filled triangles), IVMB (cyan x), valerate (orange open square), and isocaproate (red open diamonds) concentrations are shown. Data are presented as an average of three replicates, and error bars are ± standard deviation.

Table 2. Metabolic efficiency of *Clostridioides difficile* (96896) cultured at 39 °C (10% *v*/*v*) measured as change (0–24 h) in optical density (OD_{600}) per change in total quantified catabolic products (ammonia and fermentation acids) represented as the average of three trials.

Media Type	Metabolic Efficiency		
	ΔOD_{600}	*ΔmM Total Catabolic Products	ΔOD/ΔmM Total Catabolic Products
RCM	2.74	41.4	0.066
T	0.77	37.81	0.020
T+G	1.42	54.74	0.026
CAA+G	0.053	6.81	0.008
CAA	0.055	3.41	0.016

*ΔmM total catabolic products per media type = (ΔNH_3) + (Δ lactate) + (Δ formate) + (Δ acetate) + (Δ butyrate) + (Δ succinate) + (Δ IVMB) + (Δ valerate) + (Δ isocaproate).

3.4. Sporulation

After 48 h of incubation at 39 °C, all strains of *C. difficile* were visually inspected for the presence of spores under a light microscope at 100× magnification. *C. difficile* is rod-shaped and a sporulating cell adopts a club-like appearance where the spore is phase-contrasted with the surrounding cell and background. The production of spores was identified in some cells of strain 9689 in RCM after 48 h of incubation, while the presence of spores for the other strains and other media types were more difficult to discern visually. Following confirmation that the type strain (9689) in the ATCC recommended growth medium (RCM) had begun to sporulate, all tubes were inoculated into fresh RCM and pasteurized. RCM was selected as the growth medium following pasteurization due to its substrate richness and the prolific growth of vegetative *C. difficile* identified in Figure 1. Following 10 days of incubation at 39 °C, the tubes were visually inspected for growth (turbidity). All strains of *C. difficile* tested (9689, 43597, and BAA-1870) yielded visual signs of growth in all media

types (RCM, CAA, CAA+G, T, and T+G), indicating that all cultures after 48 h produced viable spores regardless of substrate.

4. Discussion

C. difficile has been isolated and grown for identification purposes in clinical and food safety testing as well as for the study of toxin formation/release [12,15,24]. The media types required for these standard uses can generally be purchased pre-made and are often supplemented with antibiotics to select for the growth of *C. difficile*, for example: CDMN (TCDMN), BHI (TBHI, TBHICC, etc.), and CCFN or CCFA (TCCFN, TCCFA) [12,13,16]. The media used for isolation and enrichment of *C. difficile*, and other bacteria, all possess the same components: water, a carbon and nitrogen source (often doubling as sources of vitamins, e.g., yeast extract), as well as a mineral source [25]. Water is necessary in media in that it plays a fundamental role in solubilizing nutrients and facilitating hydrolysis reactions. Carbon sources serve as catabolic substrates for energy as well as anabolic components that make up the cell [26]. A source of nitrogen is also crucial for bacterial growth as nitrogen is necessary for the synthesis of amino acids, DNA, RNA, and proteins, among other molecules crucial to cellular function [27]. Organic compounds such as proteose-peptone or tryptone are common sources of nitrogen and carbon in growth media. Lastly, minerals are necessary for the formation of critical metal-containing enzymes, as they act as an energy source through redox reactions by serving as electron sinks or sources, among other roles [28]. Common mineral salts in growth media can include phosphates, sulfates, and magnesium. The Hungate-style media employed in this study possessed the basic requirements, in theory, to support microbial growth. However, the objective was to determine which, if any, substrates supported the growth of *C. difficile* more than others and additionally how those variable substrates affected microbial metabolism as a means to demonstrate the benefits of substrate manipulation in microbial media for a pathogen of interest.

The American Type Culture Collection recommends two types of media for the growth of *Clostridioides difficile* strain 9689 in a laboratory setting: 1. Reinforced Clostridium Media (ATCC medium 2107) or 2. Tryptic Soy Medium with 5% Defibrinated Sheep Blood (ATCC medium 260) [29]. We elected to use the reinforced clostridium medium (RCM) as a pre-made media source, which contains several sources of carbon, nitrogen, vitamins, and minerals. Tryptone, beef extract, and dextrose/glucose serve as sources of carbon, nitrogen, and some vitamins, yeast extract which supplies a source of B vitamins, cysteine HCl as a reducing agent, and sodium acetate whose original purpose in the medium was to act as a buffer but can also serve as a substrate in the Wood–Ljungdahl pathway [17]. Indeed, we found that the sodium acetate in the medium was not metabolically inert but instead that about half of the ~40 mM acetate was utilized by 9689 over 24 h. *C. difficile* consistently grew to greater optical density in RCM than in the other media types (3.0 versus < 1.5 OD_{600}, respectively) (Figure 1). Not only did 9689 grow to greater density in RCM but it also appeared to do so with greater metabolic efficiency than in the other media types (Table 2). However, 9689 did not appear to perform considerable amino acid fermentations (including Stickland reactions) as evidenced by the little ammonia (NH_3) it produced after 24 h (~5 mM) (Figure 2). However, the increased presence of valerate and isocaproate after 24 h indicates that some amino acid fermentation occurred in the cells grown in RCM media (Table 1, Figure 3a). Generally, 9689 seems to have largely utilized its partial glycolysis cycle (full Embden–Meyerhof–Parnas pathway with partial Krebs) for its metabolism. Evidenced by the large production of butyrate (~25 mM) and formate, which was detectable in smaller amounts, following the clear utilization of glucose in the media (Figure 3a). This metabolic route would prove advantageous to 9689 as it would allow for the generation of ATP from butyrate and regeneration of reducing equivalents from further metabolism of formate to hydrogen [17]. It seems likely that RCM led to more luxuriant growth because it is rich in several substrates. However, it is important to note that RCM

differs from the other media types in other ways, including pH, phosphate vs. carbonate buffer, and N_2 vs. CO_2 saturation.

Comparatively, when *C. difficile* was grown in T and T+G media types, it appeared to perform amino acid fermentation more than in RCM. When grown in T and T+G Hungate-style media, 9689 produced the most NH_3 of any other media type used (~25 and ~17 mM, respectively) (Figure 2). Similar to RCM media, 9689 produced both valerate and isocaproate; however, it produced quantifiable amounts of IVMB as products of amino acid fermentation as well (Table 1). However, in T media, 9689 produced less valerate than in RCM media, but the same amount of isocaproate was produced across the three media types (Table 1). Similar to the fermentation acids originating from glucose metabolism identified in RCM media, 9689 also produced formate (significantly more) and butyrate (significantly less) in T+G media (Table 1). Instead of producing greater quantities of butyrate, as in RCM media, 9689 further metabolized its carbohydrate substrate, glucose, to acetate (16.24 mM), which would serve as a means to generate ATP for the organism (Table 1) [30]. Similarly, in T media, 9689 produced acetate as well, but it did not produce any quantifiable formate or butyrate (Table 2). Whether glucose was present in tryptone/trypticase (T+G) media or not (T), metabolic efficiency remained relatively consistent (0.026 and 0.020, respectively) (Table 2).

While *C. difficile* grew reliably in RCM, T, and T+G media types, it did not grow reliably, nor did it appear to perform much metabolism when inoculated in CAA or CAA+G media (Figures 1 and 2). When grown in both CAA and CAA+G, 9689 produced < 5 mM NH_3, indicating minimal amino acid fermentation when compared to T and T+G media types (Figure 2). Additionally, in CAA+G media, 9689 produced minimal quantities of fermentation acids (acetate—3.27 mM and isocaproate—1.32 mM) (Table 1). Fermentation acids from CAA were not analyzed due to the absence of a carbohydrate source for glycolysis and the minimal production of NH_3. The negligible growth and limited metabolism of 9689 in CAA and CAA+G compared to T and T+G media is presumably due to the different amino acid and vitamin compositions. However, it is important to note that despite the growth and metabolic restrictions of the CAA media types, *C. difficile* is still able to persist through successful sporulation similarly to the other growth media. Casamino acids are a hydrochloride acid hydrolysate of casein where the acid treatment destroys the tryptophan and vitamins present, whereas tryptone is simply the assortment of peptides produced from the digestion of casein sans acid treatment. Interestingly, research has previously reported reliable growth and fermentation of *C. difficile* strains in media supplemented with casamino acids [31]. However, it is important to note that in the study, casamino acids were not used as the primary substrate source in the media, which was *Clostridium difficile* minimal media (CDMM), and was supplemented with glucose, tryptophan, sodium hydrogen selenite, and biotin [32]. This suggests that when *C. difficile* is grown on CAA or CAA+G alone it is not enough to facilitate continued growth or metabolism but when instead CAA is added as a supplement to an already rich medium *C. difficile* will grow reliably. Growth in such a medium by *C. difficile* may occur as a result of mixed Stickland-type fermentations (carbohydrate H-donors and amino acid H-acceptors) between glucose and existing H-acceptor amino acids such as L-proline or leucine, both of which are present in casamino acids, as demonstrated in previous metabolic work with *C. sporogenes* [33]. This is further supported through work carried out by [34], where they reported that *C. difficile* will not grow following the sudden omission of L-proline/leucine from the medium but can be adapted to grow without a preferred Stickland H-acceptor through increased usage of the Wood–Ljungdahl pathway in the presence of glucose.

These data demonstrate that growth substrate affects the metabolism of *C. difficile* and ultimately impacts the metabolites it produces as a result of the fermentations performed. This type of substrate manipulation allows for different avenues of metabolism to be explored for microorganisms in general but also impacts product formation in potentially predictable ways. There are clear advantages to using ready-made media powders as is the case in clinical settings and food safety testing. However, custom, defined media types

are advantageous for the study of physiology and metabolism. *C. difficile* grows in the Hungate-style media used by rumen microbiologists when the correct growth substrates are included.

Author Contributions: Conceptualization, J.E.L. and M.D.F.; Methodology, J.E.L., J.L.F. and M.D.F.; Formal analysis, J.E.L.; Writing – original draft, J.E.L.; Writing – review & editing, J.E.L.; J.E.L. and M.D.F.; Supervision, M.D.F.; Funding acquisition, M.D.F. All authors have read and agreed to the published version of the manuscript.

Funding: J.E.L. was supported by an appointment to the Agricultural Research Service (ARS) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) under DOE contract number DE-SC0014664. M.D.F. and J.L.F. were supported by the USDA, Agricultural Research Service National Program NP-215, Grass, Forage and Rangeland Agroecosystems.

Data Availability Statement: The data underlying this article are available within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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